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Escola de Engenharia

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Elucidation of the molecular mechanisms underlying the cytotoxic effect of recombinant frutalin in human tumor cells

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e da
Professora Doutora Lucília Saraiva

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Abstract

Frutalin is the α-D-galactose-binding jacalin-related lectin isolated from breadfruit seeds (*Artocarpus incisa)*. Frutalin has been previously produced in *Pichia pastoris* and in *Escherichia coli* in order to overcome the limitations associated with the extraction from its natural source. A previous study also showed that recombinant frutalin expressed in *Pichia pastoris* and purified by size-exclusion chromatography (SEC) induces apoptosis in HeLa tumoral cells. Nevertheless, the molecular mechanism of apoptosis induction triggered by frutalin has not been studied.

Therefore, with the present work it was intended to elucidate the molecular mechanism involved in apoptosis induction by the recombinant frutalin expressed in *Pichia pastoris* and purified by SEC. To achieve such a goal, the modulatory effect of frutalin on main regulatory proteins of apoptosis, such as the executioner members of the caspase family (caspases-3, -6 and -7) and the p53 family members was studied using yeast-based assays. The results obtained showed that the p53 family proteins (p53, p63, p73 and DN) are not direct targets of frutalin. Moreover, using human tumor cells with (HCT116 $p53^{+/+}$) and without (HCT116 $p53^{-/-}$) wild-type p53, we confirmed that frutalin induces apoptosis by a p53-independent pathway. Concerning the procaspases-3, -6 and -7, the results obtained suggest that frutalin induces apoptosis by a caspase-dependent pathway.

In addition, different molecules of frutalin expressed in a different expression system (*E. coli*) and/or purified by a different methodology (hydrophobic interaction chromatography; HIC) were tested for its anti-proliferative activity in order to see if one could improve the production and purification process. Thus, the activity of frutalin produced in the bacteria *Escherichia coli* and in the yeast *Pichia pastoris* purified by HIC was also evaluated regarding its effect on the proliferation of HCT116 $p53^{+/+}$ tumor cells. The frutalin expressed in *E. coli* did not inhibit cell proliferation. Additionally, frutalin expressed in *P. pastoris* and purified by HIC, resulted in two different samples suggesting that one is partially glycosylated and the other is non-glycosylated. The sample partially glycosylated also showed an inhibitory of proliferation of HCT116 $p53^{+/+}$ tumor cells. However, this activity was less potent than the one obtained with frutalin from *P. pastoris* and purified by SEC. The sample non-glycosylated had no anti-proliferative effects. The same result was obtained for frutalin expressed in *E. coli,* suggesting that glycosylation affects the biological activity of frutalin.

In conclusion, trials to obtain the recombinant frutalin in a more straightforward production/purification process were ineffective as alternative systems, because the anti-proliferative activity of frutalin was compromised. Frutalin produced from *Pichia pastoris* and purified by SEC has, nevertheless, a potent anti-proliferative effect on HCT116 tumor cells and induces apoptosis through a caspase-dependent pathway.

Resumo

Frutalina é uma lectina α-D-galactose-ligante jacalina-relacionada isolada nas sementes da planta fruta-pão (*Artocarpus incisa*). A frutalina foi produzida, anteriormente, em *Pichia pastoris* e em *Escherichia coli* de modo a superar limitações associadas à sua extração da fonte natural. Um estudo anterior mostrou que a frutalina expressa em *Pichia pastoris* e purificada por cromatografia de exclusão de peso molecular (SEC) induz a apoptose em células tumorais humanas. Todavia, o mecanismo molecular de indução da apoptose pela frutalina ainda não foi estudado.

Assim, com o presente trabalho pretende-se elucidar o mecanismo molecular envolvido na indução da apoptose pela frutalina expressa em *P. pastoris* e purificada por SEC. Para atingir este objetivo foi estudado, através da utilização de um modelo celular de levedura, o efeito modulador da frutalina nas principais proteínas reguladoras da apoptose como membros da família de caspases executoras (caspases-3, -6 and -7) e membros da família da proteína p53 (p53, p63, p73 e DN). Os resultados obtidos mostraram que os membros da família da proteína p53 não são alvos diretos da frutalina. Além disso confirmou-se, através da utilização de células tumorais humanas com (HCT116 $p53^{+/+}$) e sem (HCT116 $p53^{-/-}$) a forma nativa da p53, que a frutalina induz a apoptose por uma via independente da p53. Relativamente aos resultados obtidos para as procaspases-3, -6 e -7, estes sugerem que a frutalina induz a apoptose por uma via dependente das caspases.

Foi testada, também, a atividade anti-proliferativa de diferentes moléculas de frutalina expressa num sistema de expressão diferente (*E. coli*) e/ou purificada por uma metodologia diferente (cromatografia de interação hidrofóbica; HIC), a fim de se verificar se poderiam melhorar o processo de produção e purificação. Assim, foi avaliado o efeito da frutalina produzida na bactéria *Escherichia coli* e na levedura *Pichia pastoris* purificada por HIC na proliferação de células tumorais HCT116 $p53^{+/+}$. A frutalina expressa em *E. coli* não inibiu a proliferação celular. Adicionalmente, a frutalina expressa em *Pichia pastoris* e purificada por HIC resultou em duas amostras diferentes sugerindo que uma é parcialmente glicosilada e outra não é glicosilada. A amostra parcialmente glicosilada mostrou ter efeito na inibição da proliferação das células tumorais HCT116 $p53^{+/+}$. No entanto, esta atividade é menos potente do que a obtida com a frutalina expressa em *P. pastoris* e purificada por SEC. A amostra não glicosilada não teve efeito na inibição da proliferação celular. O mesmo resultado foi obtido para a frutalina expressa em *E. coli* sugerindo que a glicosilação afeta a atividade biológica da frutalina.

Em conclusão, os ensaios para obter a frutalina recombinante através de um processo de produção/purificação mais simples foram ineficazes como sistemas alternativos, uma vez que comprometeram a atividade anti-proliferativa da frutalina. A frutalina produzida por *Pichia pastoris* e

purificada por cromatografia de exclusão de peso molecular tem, no entanto, um potente efeito anti-proliferativo nas células tumorais HCT116 e induz a apoptose por uma via dependente das caspases.

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Abbreviations

BPH	Benign prostate hyperplasia
BMM	Minimal methanol medium
BMG	Minimal glycerol medium
BSA	Bovine serum albumin
CFU	Colony-Forming Unit
Cyt c	Cytochrome c
Con A	Concanavalin A
CV	Column volumes
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DR	Death receptor
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
Fh8	*Fasciola hepatica* 8-kDa protein
FITC-VAD-fmk	Fluorescein isothiocyanate conjugate of z-VAD-fmk
FTL	Frutalin
gJRLs	Galactose-specific jacalin-related lectins
HeLa	Human cervical cancer cells
HIC	Hydrophobic interaction chromatography
His	Histidine
IMAC-Ni	Immobilized nickel ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JRLs	Jacalin-related lectins

kDa	kilodalton
KML-C	Korean mistletoe
LB	Luria-Bertani broth medium
MEC	Mucoepidermoid carcinoma
Met	Methionine
mJRLs	Mannose-specific jacalin-related lectins
MOMP	Mitochondrial outer membrane permeabilization
N	Asparagine
OD	Optical density
PBS	Phosphate Buffered Saline
PCL	*Polygonatum cyrtonema* lectin
PMSF	Phenylmethanesulfonyl fluoride
POL	*Polygonatum odoratum* lectin
ROS	Reactive oxygen species
S	Serine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SFL	Lectin from Sophora flavescens
S.E.M.	Standard error
SRB	Sulfurhodamine B
T	Threonine
TBS	Tris- buffered saline
TCA	Trichloroacetic acid
UEA-I	*Ulex europeans* I
Z-VAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Chapter 1
General Introduction

1.1 Plant lectins

Lectins are a heterogeneous group of proteins with at least one non-catalytic domain that selectively recognizes, and reversibly binds to, specific free sugars or glycans present on glycoproteins and glycolipids without altering the structure of the carbohydrates [1]. These are distributed in nature namely in plants, viruses, animals, bacteria, vertebrates and invertebrates [1, 2]. The first description of plant lectins dates back to the late nineteenth century when Peter Stillmark initiated the study of proteins derived from plants that have demonstrated the ability to agglutinate erythrocytes [3, 4]. In 1988, in his doctoral thesis, he described the properties of agglutination of proteins that had been extracted and partially purified from castor seeds (*Ricinus communis*) denominated ricin. Table 1 describes the main events, over time, in relation to plant lectins.

Table 1. Major historic events of plant lectins (adapted from [5])

Year	Description
1888	Detection of erythrocyte agglutination by a toxic protein fraction from castor seeds (termed ricin) (P. Stillmark)
1891	Toxic plant agglutinins applied as model antigens (P. Ehrlich)
1898	Introduction of the term "haemagglutinin" or "phytohaemagglutinin" for plant proteins that agglutinate red blood cells (M. Elfstrand)
1907/1909	Detection of non-toxic agglutinins in plants, of their nature as proteins and of "deagglutination" of erythrocytes by hog gastric mucin (K. Landsteiner, H. Raubitschek)
1919	Crystallization of a globulin from jack bean, concanavalin A (J. B. Sumner)
1935/1936	Concanavalin A identified as jack bean haemagglutinin (J. B. Sumner)
1947/1948	Detection of plant agglutinins specific for the human histo-blood group A (W. C. Boyd; K. O. Renkonen)
1952	Use of lectins and glycosidases to prove that blood group antigens are sugars and to deduce the structures of the antigens (W. M. Watkins, W. T. J. Morgan)
1954	Introduction of the term "lectin" for plant agglutinins (W. C. Boyd)
1960	Detection of the mitogenic potency of lectins toward lymphocytes (P. C. Nowell)
1963	Introduction of affinity chromatography for the isolation of lectins (I. J. Goldstein, B. B. L. Agrawal)
1972	Determination of the amino acid sequence and the three-dimensional structure of a lectin, concanavalin A (G. M. Edelman, K. O. Hardman, C. F. Ainsworth et al.)
1983	Detection of the insecticidal action of a plant lectin (L. L. Murdock)
1984	Isolation of lectins from tumors (H.-J. Gabius; R. Lotan, A. Raz)
1989	Detection of the fungicidal action of a plant lectin (W. J. Peumans)
1995	Structural analysis of a lectin-ligand complex in solution by NMR spectroscopy (J. Barbero and colleagues)
2001-2005	Development of glycan/lectin microarrays for specificity analysis of lectins/ structural analysis of glycans and glycoproteomics (various laboratories worldwide)

The proteins that have ability to agglutinate other cells began to be called agglutinin and phytohemagglutinins [4, 6]. Later, in 1954, Boyd proposed the name lectin (derived from the Latin "legere", which means "to select", "who chooses") due to its ability to agglutinate erythrocytes [2, 3, 5]. Although nowadays the term lectins is the most commonly used, the term hemagglutinins and agglutinins can also be used to denominate them. This denomination is considered by some authors as the most accurate, since it refers to the ability of the proteins to agglutinate erythrocytes and other cell types [3, 4].

Lectins were initially defined as a group of "carbohydrate-binding proteins of non-immune origin that agglutinate cells and/or precipitate glycoconjugates". This definition has been accepted, albeit with limitations, by the Nomenclature Committee of the International Union of Biochemistry [4]. One limitation of this definition is that excludes many lectins. This is because this definition only refers to multivalent carbohydrate-binding proteins excluding proteins which have a single carbohydrate-binding domain, which, do not have the ability to agglutinate cells or precipitate glycoconjugates [4]. In 1995, Peumans and Van Damme proposed a new definition for lectins: "all plant proteins that possess at least one noncatalytic domain that binds reversibly to a specific mono- or oligosaccharide" [3]. The definition that has been considered the most appropriated is that lectins are heterogeneous group of proteins with at least one non-catalytic domain that selectively recognizes, and reversibly binds to, specific free sugars or glycans present on glycoproteins and glycolipids without altering the structure of the carbohydrates [1].

Thereafter it was proposed a subdivision of lectins on "merolectins", "hololectins", "chimerolectins" and "superlectins" (Figure 1) according to the overall structure of the mature lectins [4].

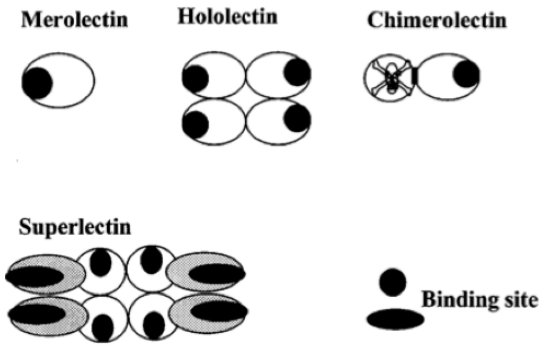

Figure 1. Schematic representation of merolectin, hololectin, chimerolectin and superlectin (adapted from [4]).

Merolectins only have a single domain binding to carbohydrates. Therefore, they are unable to agglutinate cells and precipitate glycoconjugates. Hololectins have two or more carbohydrate-binding domain wherein at least two of the binding domains are identical or homologs and bind to the same sugar

or with a similar structure. Hololectins are di- or multivalent and unlike merolectins have the ability to agglutinate cells and/or precipitated glycoconjugates. Chimerolectins exhibit one or more carbohydrate binding domain(s) tandemly arrayed to an independent domain that can have a well-defined enzyme activity or other biological activity. This activity should be independent of carbohydrate binding domains, and the number of these domains determines the behavior of these lectins. Thus, if they only have one binding domain, they behave as merolectins, whereas when they have more than one domain they behave as hololectins. Finally, superlectins are formed as hololectins with at least two carbohydrate binding domains. However, unlike hololectins these domains are structurally and functionally different, and can recognize different sugars structures [3, 4].

Lectins widely differ from each other relatively to their biochemical/physicochemical properties, affinity for carbohydrate, molecular structure and biological activities [3]. Thus, Peumans and Van Damme [3] classified lectins into seven distinct families in accordance with the molecular structure and specificity of the carbohydrate. Therefore, lectins of a given family share common features between them. Within the seven families of lectins, four are numerous, including the legume lectins, the monocot mannose-binding lectins, the chitin-binding lectins, and the type 2 ribosome-inactivating proteins (RIPs). The jacalin-related lectins, the amaranthin lectin family and the *Cucurbitaceae phloem* lectins are considered small families [4]. Lastly, plant lectins have been divided into twelve different families based on carbohydrate binding domain (e.g. jacalins, proteins with legume lectins domains and ricin-B family) and according to molecular structures and amino-acid sequences [1].

1.1.1 Potential biomedical applications of plant lectins

As mentioned before, most lectins have the ability to agglutinate other cells. This effect is directly related to their ability to recognize and to bind reversibly to specific sugar structures, establishing a connection protein-carbohydrate. This feature allows lectins mediate a variety of biological processes, such as cell-cell communication, differentiation and cancer metastasis, as well as innate immune responses [1, 2].

Lectins are used as research tools in many scientific areas, namely in biochemistry, immunology, cell biology and even in the diagnosis and treatment of cancer [7].

The use of lectins in the diagnosis and treatment of cancer, a major disease of the XXI century, has contributed to the growing interest in the study of these proteins [8]. Lectins have contributed to the

recognition of surface markers, mitogenic stimulation, cell adhesion and localization, increase of host immune defense, cytotoxicity and apoptosis [9–11].

Several studies have indicated that cancer cells have an aberrant glycosylation [12]. Once lectins bind to specific carbohydrates, they can be used to detect aberrant glycosylation. One study showed that the lectins Concanavalin A (Con A) and *Ulex europeus* I (UEA-I) can be used as histochemical biomarkers of mucoepidermoid carcinoma (MEC) in the most common malignant salivary tumor, which can be classified as low, intermediate or high grade. Con A binds to the neoplastic cells of the three degrees of severity. UEA-I is connected more strongly to cells of intermediate grade and more weakly to the other degrees [13].

Some lectins have showed the ability to induce cell recruitment, lymphocyte proliferation, and cytokine production and to possess immunomodulatory activity. Lectins (KML-C) from *Korean mistletoe* (*Viscum album coloratum*) showed differentially modulated macrophage-mediated immune responses. Moreover, they increased expression of various cytokines (IL-3, IL-23 and TNF-a) and reactive oxygen species (ROS) production [14].

1.1.2 Jacalin-related lectins (JRLs)

Jacalin-related lectins are considered a small family, when compared with other existing families of lectins. JRLs are divided into two subfamilies according to their specificity to carbohydrate. This family is subdivided into lectins which have an affinity to bind to the galactose (gJRLs) and mannose/maltose (mJRLs) [4, 15]. The “galactose-specific” and “mannose-specific” jacalin-related lectins beyond the differences on the specificity to carbohydrate, they also differ in their biosynthesis, processing, topogenesis and intracellular location [16].

Concerning to the biosynthesis differences between gJRLs and mJRLs, in the case of gJRLs it undergo proteolytic cleavage of the precursor polypeptide, resulting in two polypeptide chains, a heavy chain (α) and a light chain (β) of 133 and 20 amino acids, respectively [4, 15, 16]. Comparatively, the mJRLs does not undergo proteolytic modification, consisting only by uncleaved protomers with approximately 150 amino acids [15, 16].

There are evidence that the mannose-specific jacalin-related proteins are located in the cytoplasm, where they are synthesized [16]. These are not subject to any post-translational modifications and the mature polypeptides of the mJRLs correspond to the entire open reading frame of their corresponding

genes [4, 16]. As a cleavage of protomer has not been found, the mJRLs have an extra loop, which makes the binding site inaccessible to galactose [17].

Jacalin, a "galactose-specific" jacalin-related lectin is synthesized in the endoplasmic reticulum [16] as preprojacalin consisting of a signal peptide of 21 amino acid residues followed by a propeptide of 39 amino acid residues, a β chain with 20 amino acid residues, a linker tetrapeptide (TSSN), and lastly, a α chain with 133 amino acid residues. Its co-translational processing involves the removal of a signal peptide and its partial glycosylation, given rise to the propeptide that is transported to the Golgi complex (Figure 2) [4, 16, 17]. Subsequently, the propeptide is cleaved at three different locations, with the removal of the N-terminal propeptide and of the linker tetrapeptide. Thus, the mature jacalin is constituted by ta N-terminal β chain and a C-terminal α chain with 20 and 133 amino acid residues, respectively, being located in the vacuole. It is possible that its processing be similar to other jacalin lectins from *Artocarpus* plant, as well as from plant *Maclura pomifera* [4, 16].

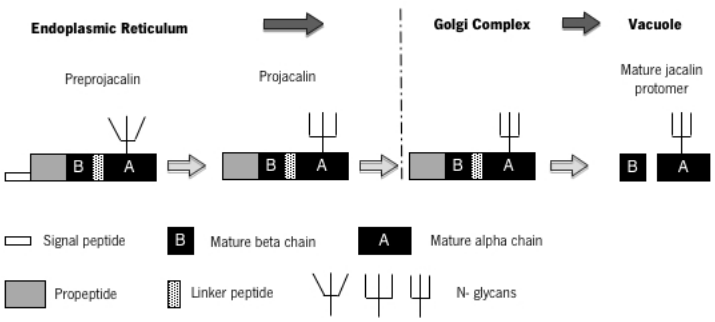

Figure 2. Schematic representation of molecular structure, biosynthesis and processing of "galactose-specific" jacalin-related lectins (adapted from [4]).

1.2 The frutalin lectin

Frutalin is a lectin isolated from the seeds of the *Artocarpus incisa* plant, commonly known as breadfruit. This plant belongs to the *Moracae* family, and it can be found in areas with a humid tropical climate. In Brazil, it is possible to find two different types of this plant, the *apyrena* (*non-seminífera*), without seeds, and the one that has seeds (*seminífera*) [18].

The frutalin is a tetrameric partially *N*-glycosylated protein which specifically recognizes α-D -galactose residues [18]. It belongs to the jacalin-related lectins family, specifically to the subfamily "galactose-specific" jacalin-related lectins. Frutalin has many structural and functional similarities with

jacalin, found in *Artocarpus integrifolia* plant that belongs to the same family as *Artocarpus Incisa*, *Moracae* family [18, 19]. As previously described, frutalin is a tetrameric molecule of approximately 48-49 kDa, consisting of four monomers linked by non-covalent bonds. Each monomer consists on a heavy chain (α) of 133 amino acids non-covalently bound to a light chain (β) of 20 amino acids. Frutalin presents predominantly a β sheet conformation and contains four binding sites for D-galactose, being therefore considered, according to its structure, a "hololectin" [17, 18, 20].

Immunologic studies showed that frutalin is a potent mitogenic activator of human lymphocytes and it has the ability to induce *in vivo* and *in vitro* neutrophil migration [19, 21].

1.3 Heterologous expression of frutalin

The use of recombinant lectins as potential therapeutic and diagnostic agents makes important to reduce the heterogeneity of properties resulting from the different lectins isolated from natural sources. In fact, lectins may have different isoforms that can lead to different specificities to carbohydrates. The heterologous expression and production of recombinant proteins reveals to be an important to overcome the limitations mentioned above. Additionally, the purification of lectins from its natural source can be a time-consuming process and with a low yield. This is also other limitation that can be overcome by the expression and production of these recombinant proteins. Additionally, through the heterologous expression and production of lectins is possible to obtain larger amounts of lectins with high levels of purity and with defined amino acid sequence, in a shorter period of time, when compared to the native lectins system. Therefore the properties of lectins are also more controlled [1, 22]. Thus, the use of heterologous expression systems have considerable advantages for the production of proteins of pharmaceutical interest [23], particularly for the study of cellular processes [24].

1.3.1 *Pichia pastoris* and *E. coli* as expression system

The ideal system should be able to express proteins with the lowest possible cost and as authentic as possible [25]. The expression system using bacterium *E. coli* and the yeast *P. pastoris* are, currently, the two most used expression systems [26, 27].

Initially, *E. coli* was the most used production system, due to extensive knowledge on its genetics, easy of manipulation, low generation time and high product yields [23, 28]. However, have significant limitations to the expression of recombinant protein using *E. coli* as an expression host. *E. coli* is unable to perform the post-translational modifications such as glycosylation or disulfide bond formation. These

modifications can affect the bioactivity, function, structure, solubility, stability, half-life, protease resistance, and compartmentalization of the functional proteins [27]. Despite of this, many proteins (e.g. IFN -α, -β and -γ, interleukin-2) that are non-glycosylated after expression in *E. coli* (glycosylated in their natural human forms) retain their biological activity. Another disadvantage of *E. coli* as an expression system is that most of proteins are produced in inclusion bodies and are often inactive, insoluble and require refolding [26].

New strategies have been developed in order to overcome some limitations, such as the solubility of the proteins expressed in *E. coli*. One example of a reliant strategy was the fusion proteins technology. Fusion partners (e.g. Fh8 tag), when fused with the protein of interest, can increase production yields, protein solubility and can be used (e.g. His tag) in the purification process [27, 29]. These limitations, in association with the high number and complexity of recombinant proteins to be expressed, led to the use of other hosts besides the *E. coli* in heterologous expression systems. Although the limitations exposed above *E. coli* remains one of the preferred hosts (host for recombinant protein expression) [30].

Pichia pastoris, the methylotrophic yeast, can overcome some of the limitations presented by the *E. coli* expression system. It is capable of post-translational modifications, namely to carry out proteins glycosylation's and to produce disulfide bonds [26]. These are major advantages of the *P. pastoris* over *E.coli*. Furthermore, production yields can be higher than *E. coli*. However, it may be necessary to optimize the growth conditions [26, 27]. Another advantage of the methylotrophic *P. pastoris* when compared to other organisms including *E. coli*, is its ability to secrete proteins efficiently. In spite of this, as a host for heterologous expression, *Pichia pastoris* also present some disadvantages. For example, *P. pastoris* can not produce proteins that require the assistance of folding chaperones. [26]. Table 2 describes the main advantages and disadvantages of the heterologous expression system *E. coli* and *P. pastoris*.

Table 2. Advantages and disadvantages of *E. coli* and *P. pastoris* expression systems (adapted from [1])

Host system	Advantages	Disadvantages
E. coli	Rapid expression High yields Genetically manipulate Inexpensive Mass production fast and cost-effective	Proteins with disulfide bonds difficult to express Produce unglycosylated proteins Proteins produced with endotoxins Proteins produced as inclusion bodies, are inactive and usually require refolding.
P. pastoris	High yield Stable production strains Durability Cost effective High density growth High productivity Stability proteins Rapid growth in chemically defined media Product processing similar to mammalian cells Can handle S–S rich proteins, assist protein folding and glycosylate proteins	Fermentation require for very high yield Growth conditions may require optimization Refolding may be required

1.3.2 Recombinant frutalin

Recombinant frutalin was expressed in a recombinant form in yeast *Pichia pastoris* and in bacteria *Escherichia coli* [29, 31, 32]. However, recombinant frutalin expressed in *P. pastoris* and *E. coli* exhibits differences between them. One major difference between them is the glycosylation. The frutalin expressed in *Pichia pastoris* is partially *N*-glycosylated whereas the frutalin expressed in *E. coli* is non-glycosylated. Furthermore, frutalin in *E. coli* showed ability to agglutinate rabbit erythrocytes contrary to frutalin in *P. pastoris* that did not showed this ability [31, 32].

Additionally, differences between recombinant and native frutalin can also be found. In fact, during the processing in *P. pastoris* and in *E.coli,* the cleavage of linker tetrapeptide (T-S-S-N) does not occur in recombinant frutalin. Therefore, in recombinant frutalin, the α and β chains are not independent, being expressed as a single chain protein. Although the differences between recombinant and native frutalin, the ability to bind to Me-α–galactose was retained by the recombinant frutalin but with lesser affinity than native frutalin [31, 32].

Recombinant frutalin expressed in *E. coli* was mainly produced as insoluble protein [32] and in order to increase the amount of frutalin, a fusion partners was used to increase production yields, promote its solubility and help on its purification. The fusion of frutalin with the Fh8 tag resulted in an increase of soluble expression and His_6 tag was used for help on its purification [29, 32].

1.3.3 Potential biomedical applications of recombinant frutalin

Oliveira et al. [12, 33] demonstrated the potentiality of recombinant frutalin expressed in *P. pastoris* as agent for the treatment and diagnosis of cancer.

Several studies showed that lectins have a promising capacity as tumor biomarkers. Recently, through immunohistochemical assays, frutalin was tested in human tissues of prostate carcinoma and benign prostate hyperplasia (BPH) as potential tumor biomarker. This study compared if native frutalin (from *Artocarpus incisa*) and recombinant frutalin were able to detect alterations in human prostate tissues. The native frutalin (n-frutalin) bound to all cases studied of prostate carcinoma and to benign prostate hyperplasia (BPH), 20 and 25 cases, respectively. Regarding to recombinant frutalin (r-frutalin), it bound to 14 of the 20 studied cases of prostate carcinoma and did not bind to none of the 25 studied cases of BPH. Recombinant frutalin showed that recognize specifically malignant cells. These differences can be due to distinct carbohydrate binding affinity. Thus, *Oliveira et al.* [12] concluded that native and recombinant frutalin could be used as histochemical biomarkers for the prostate cancer.

In another study by *Oliveira et al.* [33], it was found a cytotoxic effect of frutalin (native and recombinant) in HeLa cervical cancer cells (Figure 3). In fact, recombinant and native frutalin had similar effects in inhibiting the proliferation of HeLa cells (GI_{50} for 24 h ≈ 100 μg/mL), an effect that showed to be time- and dose-dependent.

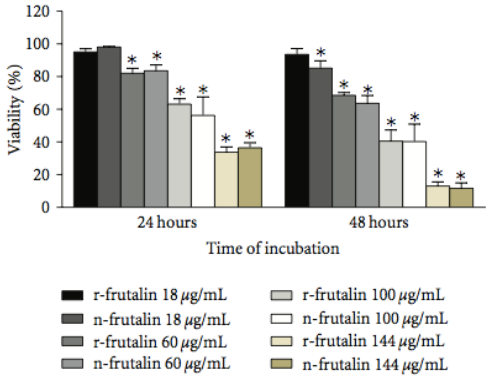

Figure 3. Effect of different concentrations of n- frutalin and r- frutalin on HeLa cells proliferation at 24 and 48 h [33].

This same study showed that the inhibition of proliferation occur due to apoptotic cell death. It was also analysed the cellular localization of each lectin studied. Interestingly, it was found that the recombinant frutalin was mainly located in the nucleus, whereas native frutalin was mainly located in the perinuclear region. This study therefore revealed a potential therapeutic application of native and recombinant frutalin against cancer [33].

1.4 Plant lectin-induced apoptosis

Apoptosis is a programmed cell death process, involved in the control of cellular proliferation and DNA damage [11]. This is characterized by typical morphological cell changes like nuclear fragmentation, chromosomal fragmentation, membrane blebbing and nuclear condensation [24, 34].

Apoptosis can be induced through several molecular pathways. The extrinsic and intrinsic pathways are the most relevant pathways in apoptosis [35]. The induction of these two apoptotic signaling pathways leads to the activation of the executioner caspases that results in the cleavage of a subset of proteins resulting in the biochemical and cellular changes typical of apoptosis [36, 37]. Caspases are a family of cysteine- dependent aspartate-specific proteases that can be divided in pro-apoptotic and pro-inflammatory subfamilies. The pro-apoptotic subfamily can be divided in activator or initiator caspases (caspase-2, -8, -9, -10 and -12) and executioner or effector caspases (caspase-3, -6 and -7) that are activated by the initiator caspases. These are synthetized as inactive preforms and stored as procaspases that under certain conditions are activated by proteolysis and become able to cleave a large set of substrates [38, 39].

The extrinsic pathway (or the death receptor pathway) involves the activation of receptors in plasma membrane by binding of ligands. These receptors are known as death receptors (DR), such as Fas and

KILLER/DR5. The activation of receptors through binding of specific ligand leads to their trimerization and consequent clustering of the intracellular death domain that results in recruitment of FAS associated with a death domain (FADD), which in turn recruits caspase-8 allowing the formation of the death-inducing-signaling-complex (DISC) [36, 37].

The intrinsic pathway (or mitochondrial pathway) involves the mitochondrial outer membrane permeabilization (MOMP), which allows a release to cytosol of pro-apoptotic proteins such as cytochrome C (cyt c) and apoptosis-inducing factor (AIF). The cytochrome c plays an important role in mitochondria-dependent apoptotic death. Upon its release it combines to the apoptosis protease-activating factor 1 (APAF-1) and the initiator caspase-9 inducing the formation of a large complex, the apoptosome, which promotes the proteolytic maturation of caspase-9. With active caspase-9, the effector caspases (caspase-3, -6 and -7) are cleaved and activated, which in turn leads to the apoptosis [36, 37].

The p53 family protein, has an important role in induction of apoptosis, namely in the intrinsic and extrinsic pathway. The p53 family protein may up-regulate several proteins involved in the intrinsic pathway. The Bcl-2 family of proteins are involved in the release of cytochrome c from the mitochondria [35]. The pro-apoptotic proteins from Bcl-2 family (e.g. Bax, Bid, Noxa, Puma) may up-regulate by p53 that results in MOMP, which in turn activate caspases leading to apoptosis [24, 36, 37]. The p53 and p73 induce the expression of p53AIP1 (p53-apoptosis inducing protein 1), which localizes to the mitochondria where it interacts with Bcl-2 to facilitate the release of cyt c and the consequent apoptosis induction through the intrinsic pathway. The p53 can also directly induce the expression of caspase-6 and Apaf-1. The p53 can upregulating two cell death receptors, KILLER/DR5 and Fas and the ligand for Fas, FasL and thus, induce the apoptosis through extrinsic pathway [35, 40].

Some of the proteins mentioned above that are involved in apoptosis such as initiator or effector caspases and p53 are lost in many cancers by inactivation or mutation of these proteins [41]. As such, induction of apoptosis by activation of these proteins is a defense against cancer and therefore an important target for cancer therapy [42–44]. Moreover, it is an important cellular homeostasis mechanism that ensures the correct development and function of multicellular organisms [45].

Different lectins induce apoptosis in different human tumor cells. The effects of plant lectins in human tumor cells are dependent of the sort of lectin and cells lineage. Their effect is time-dosage dependent. For example, *Polygonatum odoratum* lectin (POL), a mannose-binding lectin induces apoptosis

through the death-receptor apoptotic pathway by increasing the levels of FasL and Fas-Associated protein with death domain (FADD) proteins that leads to the caspase -8 activation. Moreover, POL treatment leads to cytochrome c release and subsequent activations of caspase -9 and caspase -3. Thus, POL induces apoptosis in a caspase-dependent manner [46]. *Polygonatum cyrtonema* lectin (PCL), a mannose/sialic acid-binding lectin induces, simultaneously, apoptosis and autophagy in human melanoma A375 cells. PCL binds to the mannose-containing receptor of human melanoma cell surface and it is internalized and localized on the mitochondria. PCL induced mitochondria to generate massive ROS production promotes the release of cytochrome c and activates the p38 and p53. Thus, PCL induces apoptosis via a mitochondrial ROS-p38-p53 pathway [47]. SFL, a mannose-binding lectin from *Sophora flavescens*, was also shown to induce apoptosis in human cervical cancer in a typical caspase-dependent manner by death receptor pathway [42]. Likewise, the lectin ricin induces apoptosis through activation of caspase-8 and subsequent activation of caspase -3 and -7 [48]. The Figure 4 demonstrates the plant lectin-induced apoptotic mechanisms implicated in intrinsic and extrinsic pathways.

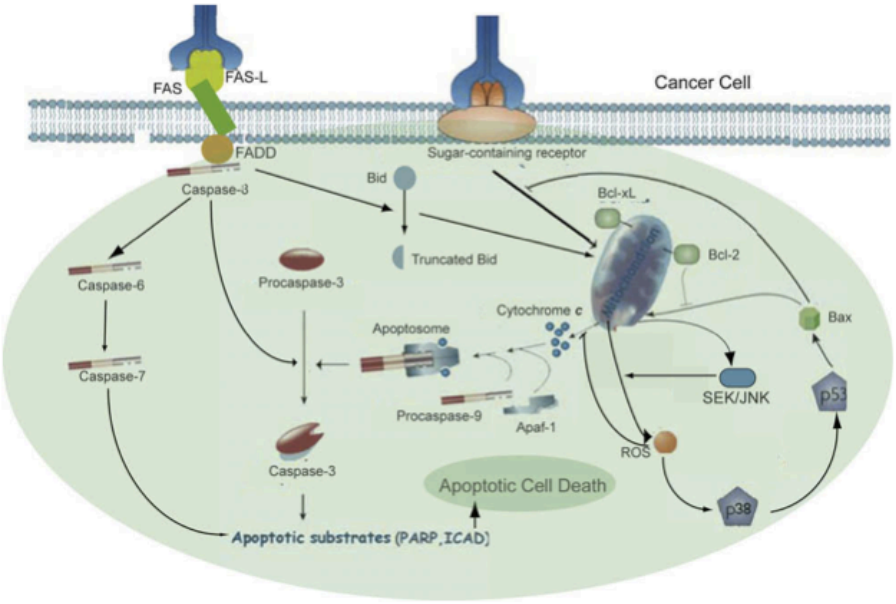

Figure 4. Plant lectin-induced apoptosis was mainly mediated by intrinsic pathway (or mitochondrial pathway) and/or extrinsic pathway (or the death receptor pathway) [46].

1.5 *Saccharomyces cerevisiae* as a model organism for molecular and pharmacological studies of human proteins

Model organisms have widely contributed to the knowledge of human cellular processes and proteins [24].

In fact, the use of *Saccharomyces cerevisiae* as a model organism has greatly contributed to the study of fields as diverse as cell metabolism, DNA replication, recombination, cell cycle, cell death, protein folding, trafficking, and organelle biogenesis [24]. *S. cerevisiae* was the first eukaryote to have its genome fully sequenced [49]. These data are easily accessible on the online dataset for yeast with genetic interactions, transcriptional changes, protein interactions, and localization information [24, 50, 51]. Additionally, it can be easily genetically manipulated, and there is a lot of information about their molecular biology and physiology. Another advantage of this yeast as a model organism is its short generation time, widely easy to cultivate and maintain [23].

Several cellular processes present in *S. cerevisiae*, such as cell cycle progression, protein secretion and apoptosis, are similar to those present in humans, thus allowing the understanding of the biological mechanism involved in human diseases. In fact, many of knowledge obtained from yeast has been transposed to the human cells [49, 52].

To establish these models for the study of heterologous proteins different approaches can be used. If the gene encoding the human protein is conserved in yeast, it is possible to study directly the function of the protein in this organism. However, if yeast does not possess orthologous of the human gene, it is necessary its heterologous expression in this organism (called "humanized yeast"). The heterologous expression in yeast of proteins involved in human diseases, has provided important information regarding the functions of these proteins (e.g. p53 and caspase family members) and about the pathobiology underlying human diseases, such as cancer [24].

1.6 Aims of the work

In a previous study, recombinant frutalin expressed in *Pichia pastoris* and purified by size-exclusion chromatography showed a strong anti-proliferative effect on HeLa cervical tumor cells. Moreover, it was shown that this effect was due to the induction of cell dead by apoptosis. However, the molecular mechanism involved in apoptosis induction triggered by this recombinant frutalin was not studied.

Thus, the main goal of this work was to elucidate the molecular mechanism involved in apoptosis induction by the recombinant frutalin expressed in *Pichia pastoris* and purified by size-exclusion chromatography.

Furthermore, it was also aim of this work to test other frutalin molecules obtained with another expression system (*Escherichia coli*) or/and another purification methodology (hydrophobic interaction chromatography). These other recombinant forms of frutalin were produced with a more straightforward production and purification process but their anti-proliferative activity in human tumor cell lines was never tested before.

Based on this, the specific aims of this work were to:

- Study the modulatory effect of frutalin expressed in *P. pastoris* and purified by size-exclusion chromatography on main regulatory proteins of apoptosis, such as the executioner members of the caspase family (caspases-3, -6 and -7) and the p53 family members using yeast- based assays;
- Study the effect of frutalin expressed in *P. pastoris* and purified by hydrophobic interaction chromatography in the proliferation of human tumor cell lines;
- Study the effect of recombinant frutalin obtained from *E. coli* and purified using affinity chromatography with nickel in the proliferation of human tumor cell lines.

Chapter 2
Materials and Methods

2.1 Systems of production and purification of recombinant proteins

2.1.1 Production of recombinant frutalin expressed in *Pichia pastoris*

Pichia pastoris KM71H/ pPICZα/ frutalin cells [31], were grown in 100 mL of buffered minimal glycerol medium (BMG) with 1% glycerol (AppliChem), 100 mM potassium phosphate (pH 6.0; AppliChem), 1.34% yeast nitrogen base with ammonium sulfate and without amino acids (Difco) and 4×10^{-5} biotin (Sigma), overnight, at 30 °C, with continuous shaking (200 rpm). The next day, cells were harvested by centrifugation at 4000 g for 10 minutes at room temperature and the resulting cell pellets were resuspended in 50 mL of fresh buffered minimal methanol medium (BMM). BMM medium has the same composition of BMG medium but glycerol is replaced with 0.5% (w/v) methanol (Biochemicals). The induction of cultures was carried out in 500 mL baffled shake flask and covered with two layers of sterile gauze. Inducible cultures were incubated at 15 °C, with continuous shaking (200 rpm) and fresh methanol was added daily, during the 4 days of induction, to a final concentration of 0.5% (v/v). After the induction period, the supernatants were separated from cell pellets by centrifugation at 4000 g, 4 °C for 10 minutes. To precipitate salts, the pH of the supernatants was increased to 7.5 by adding 10 N NaOH and removed by centrifugation twice at 4000 g, 10 minutes at 4 °C.

2.1.2 Purification of recombinant frutalin expressed in *Pichia pastoris*

The supernatants were filtered through filters of 0.2 μm, concentrated and washed with Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, 100 mM Na_2HPO_4) in 10 kDa Amicon filters (Millipore) to a final volume of 1–1.5 mL. To confirm the presence of recombinant frutalin in the samples, these were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie (described in section 2.1.5/2.1.5.1). After this, samples were purified by size-exclusion chromatography (SEC) or hydrophobic interaction chromatography (HIC). At the end of purification, samples were quantified by absorbance 280 nm, using NanoDrop 1000 (Thermo Scientific) based on molar extinction coefficient of frutalin ($\varepsilon_{frutalin}$ = 27390 M^{-1} cm^{-1}).

2.1.2.1 Size-exclusion chromatography (SEC)

SEC is a chromatography technique that separates biomolecules according to differences in their size. In this work, a Sephacryl™ S-200 HR column (GE Healthcare) was used in FPLC system (Pharmacia Biotechnology). In the first step, the column was equilibrated with PBS at a flow rate of 1 mL/min. The

sample was filtered through 0.2 μm pore size filters and applied on the column at a flow rate of 1 mL/ min and elution occurs under the same conditions. Purified samples were collected and aliquot to be analyzed by SDS-PAGE (described in section 2.1.5/2.1.5.1). The samples containing pure recombinant frutalin were concentrated in 10 kDa Amicon filters (Millipore) and then stored at –20 °C.

2.1.2.2 Hydrophobic interaction chromatography (HIC)

HIC separates biomolecules according to the differences in their hydrophobicity. In this work, a prepacked Phenyl Sepharose™ 6 fast flow High Sub column (GE Healthcare) was used operated by a peristaltic pump (Pharmacia LKB pump) and the whole procedure of purification was performed at 4 °C. Table 3 presents the description of buffers used in this purification in order to optimize the elution conditions. In the first step, column was equilibrated with 8 column volumes (CV) of buffer (start buffer) with moderately high salt concentrations. Then, the sample was filtered through 0.2 μm pore size filters and applied on the column at a flow rate of 0.5 mL/min. Then, column was washed with 8 CV of washing buffer in order to remove all unbound proteins with a flow rate of 1 mL/min. For elution of proteins, an elution buffer with a flow rate of 0.5 mL/min was used. During purification, aliquots were collected and analyzed by SDS-PAGE (described in section 2.1.5/2.1.5.2). Finally, the column used in the procedure was cleaned with 2 CV of EDTA 100 mM, following with 2 CV of water, 2 CV of 6M Guanidinium in PBS solution and at least 2 CV of water. To store the column at 4 °C, 2 CV of EtOH 20% was used.

Table 3. Buffers recipe for HIC purification assays

	Assay 1	Assay 2
Samples		Diluted 1:2 with PBS with 5 M NaCl (pH 7.4)
Start buffer	PBS (pH 7.4)	PBS with 2.5 M NaCl (pH 7.4)
Washing buffer	1. PBS 2. PBS diluted 1:2 (pH 7.4)	PBS with 2.5 M NaCl (pH 7.4)
Elution buffer	Tris 50 mM (pH 10)	Step gradient elution: 1. PBS with 2 M NaCl (pH 7.4) 2. PBS with 1.5 M NaCl (pH 7.4) 3. PBS with 1 M NaCl (pH 7.4) 4. PBS with 0.5 M NaCl (pH 7.4) 5. PBS with 0.25 M NaCl (pH 7.4) 6. PBS (pH 7.4) 7. Tris 50 mM (pH 10)

In the samples with pure frutalin buffer exchange with PD-10 Desalting Column (GE Healthcare) was performed. Thus, this column was washed with 10 mL of distilled water, following 25 mL PBS. Then,

2.5 mL of the sample was applied on the column and eluted with 3.5 mL PBS. After, samples were stored at –20 °C and the column was washed with water and stored in EtOH 20%.

2.1.3 Production of recombinant frutalin expressed in *E. coli*

E. coli Rosseta (DE3) cells, transforming with pETM11 or pETMFh8 encoding His_6-frutalin or Fh8-frutalin [29], respectively, were grown overnight at 37 °C with continuous shaking (180 rpm) in 25 mL LB medium (Applichem) supplemented with 30 mg/mL kanamycin (Sigma) and 10 mg/mL chloramphenicol (Applichem). The next day, four erlenmeyers were prepared with 250 mL of LB medium supplemented with 30 mg/mL kanamycin and 10 mg/mL chloramphenicol to make up 1 L culture. Thus, each of these erlenmeyers was inoculated with pre-culture so that the initial 0.02 optical density measured at 600 nm (OD_{600}). After inoculation, cell cultures were incubated at 37 °C with continuous shaking (180 rpm) until the OD_{600} reached a value between 0.4 - 0.6. After achieving this OD, the cell culture was maintained under stirring but at 18 °C. After 30 minutes, the expression of frutalin was induced by the addition of IPTG (Bio-Rad) to a final concentration of 0.2 mM and incubated overnight, at 18 °C with continuous shaking (180 rpm). Thereafter, cell pellet was recovered by centrifugation at 4500 rpm, for 15 min, at 4 °C and supernatant was discarded. Cell pellets were stored at –20 °C. Before and after the induction aliquots were recovered for analyze through SDS-PAGE (described in section 2.1.5/2.1.5.1).

Before the cells being purified (described in section 2.1.4) they were resuspended in 25 mL of lysis buffer (50 mM Tris pH 8.0 (JTBacker), 150 mM NaCl (AppliChem), 20 mM Imidazole (Sigma)) with 1 mM of PMSF (Sigma), 5 μL/mL DNase (Sigma) and 10 μL/mL lysozyme (Sigma). After this, cells were sonicated (Branson Sonifier 450, 6 minutes, duty cycle 50%, output 5) and then, the supernatant was harvested by centrifugation at 4 °C, 13000 g for 30 min. Supernatant was filtered through 0.45 μm pore size filters. Aliquots of total lysates and supernatant were collected for analyses by SDS-PAGE (described in section 2.1.5/2.1.5.1).

2.1.4 Purification of recombinant frutalin expressed in *E. coli*

To purify His_6-FTL and Fh8-FTL proteins immobilized nickel ion affinity chromatography (IMAC-Ni) was performed. Therefore, HisTrap column (GE Healthcare) was used operated with peristaltic pump and all the procedure was performed at 4 °C. The column was equilibrated with 4-6 CV with a binding buffer (50 mM Tris pH 8.0, 150 mM NaCl, 20 mM Imidazole) with a flow rate of 1 mL/min. Then, the samples were applied to the column with a flow rate of 0.5 mL/min (in this stage the proteins with histidines bind to

ligands, nickel ions). After this, the column was washed with 5-10 CV of washing buffer (50 mM Tris pH 8.0, 150 mM NaCl, 40 mM Imidazole) to remove unbound material with a flow rate of 1 mL/min. Then, for elution of the target protein an elution buffer with 50 mM Tris pH 8.0, 150 mM NaCl and 300 mM Imidazole was used. The purified samples of 5 mL were collected in 15 mL tubes and posteriorly analyzed by SDS-PAGE. To clean the column 2 CV of 70% EtOH, following 2 CV of distillate water were applied. Then, it was washed with 2 CV of 1M NaOH and at least 2 CV water. To store the column at 4 °C, 2 CV of 20% EtOH was applied.

After purification, it was necessary to perform the dialysis. Thus, samples were dialyzed in PBS buffer (pH 7.4), overnight, at 4 °C. These proteins were quantified by the Bradford assay with Bio-Rad protein reagent (Bio-Rad). Therefore, in 96-well plates, it was loaded 10 μL/well of protein sample with 200 μL of BioRad Protein reagent diluted 1:5. After 5-10 minutes, the absorbance at 595 nm was measured using the Biotech Synergy HT Microplate Reader. Bovine serum albumin (BSA) was used as standard.

2.1.5 SDS-PAGE

The electrophoresis under denaturing conditions, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was based on the Laemmli system [53]. The proteins were analyzed, according to this methodology, before, during and after the purification process. This system uses polyacrylamide gel with different acrylamide percentages to separate proteins based on the molecular weight. In this work, proteins were separated on 12% SDS polyacrylamide gels (SDS-PAGE). The protein molecular weight marker used in SDS-PAGE electrophoresis was PageRulerTM Unstained Broad Range Protein Ladder (5-250 kDa) from Fermentas. The composition of SDS-polyacrylamide gels is present in Table 4.

Table 4. Composition of SDS polyacrylamide gels

Reagents	Resolving gel (12%)	Stacking gel (4%)
40% Acrilamid/Bis	3 mL	0.5 mL
0.5 M Tris-HCl, pH 6.8	-	1.25 mL
1.5 M Tris-HCl, pH 8.8	2.5 mL	-
10% SDS	100 μL	50 μL
10% APS	50 μL	25 μL
TEMED	5 μL	2.5 μL
H_2O	4.35 mL	3.17 mL
Final volume	10 mL	5 mL

The samples were treated with the loading sample buffer (0.15 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) Glycerol, 25% (w/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue) and heated at 100 °C for 5-10 min. The electrophoresis run was performed at constant voltage 120 V, using the running buffer (17.7 mM Tris, 0.25 % (w/v) SDS and 0.2 M Glycine).

2.1.5.1 Coomassie blue staining method

After electrophoresis, Coomassie Blue staining method was used to stain gels. Gels were placed in distilled water and heated in microwave for 30 seconds, then placed under constant agitation during 3 minutes. This step is repeated twice with fresh distilled water. Following this, gels were placed in Coomassie Blue solution, heated in microwave for 30 seconds and then placed under constant agitation during 30 minutes. Finally, Coomassie Blue solution was removed and distilled water was added for destaining of the gel. Coomassie Blue solution was prepared dissolving 60-80 mg the Coomassie Brilliant Blue G250 in 1 L of distilled water. This solution was stirring with a magnet for 2 to 4 hours. After this, add 3 mL of concentrated HCL 37% and solution is stored at environment temperature and protected from light.

2.1.5.2 Silver nitrate staining method

The polyacrylamide gels resulting of SDS-PAGE were stained with silver nitrate to detect proteins with lower concentrations. Thus, the gels were washed with 20% ethanol for 10 minutes and washed with distilled water for 10 minutes. Then, gels were sensitized with 0.2 g/L sodium thiosulfate for 2-5 minutes. After this, the gels were washed twice, for 20 seconds with distilled water. The gels were stained with 2.0 g/L nitrate prate solution for 30 minutes and then washed with distilled water five times for 10 seconds. To develop the color, a solution with 0.07% formaldehyde (37% formaldehyde), 30 g/L potassium carbonate anhydrous and 10 mg/L sodium thiosulfate was used. To stop the staining, gels were placed in a solution stop (50 g/L tris base and 2.5% acetic acid) and stored in distilled water.

2.2 Yeast-cell based phenotypic assay

2.2.1 Plasmids

The yeast expression vectors pLS89-(*TRP1*) encoding human wild-type (wt) p53, pRS314-(*TRP1*) encoding human TAp63α, ΔNp63α and pRS314-(*TRP1*) encoding human TAp73α with *GAL1-10* inducible

promoters, were used. Yeast expression vectors pGALL-(*LEU2*) encoding human procaspase-3, procaspase-6 and procaspase-7 under a *GAL1-10* promoter were used.

2.2.2 Yeast strain, transformation and growth conditions

Saccharomyces cerevisiae strain CG379 (*α ade5 his7-2leu2-112 trp1-289α ura3-52 [Kil-O]*, Yeast Genetic Stock Center, University of California, USA) [54] was transformed using the standard lithium acetate method [55]. Yeast cells were routinely grown in minimal selective medium with 2% (w/w) glucose (Sigma), 0.7 % (w/w) yeast nitrogen base without amino acids (Difco), and all the amino acids required for yeast growth (50 μg/mL; Sigma) except tryptophan (for wt p53, TAp63α, ΔNp63α and TAp73α), or except leucine (for procaspase-3, -6 and -7). For expression of human proteins, cells were diluted to 0.05 optical density (OD_{600}) in selective induction medium, in which glucose was replaced by 2% (w/w) galactose (Sigma) and 2% (w/w) raffinose (Sigma). Yeast cells were then incubated at 30 °C, under continuous orbital shaking (200 rpm) for approximately 30 h (for wt p53, TAp63α, ΔNp63α and TAp73α) or 42 h (for procaspase-3, -6 and -7), corresponding to the time required by control yeast (transformed with the empty vectors pLS89, pRS314 or pGALL) to achieve 0.5 OD_{600}. For growth curves experiments of yeast cells expressing procaspase-3, procaspase-6 and procaspase-7, the growth of cultures was analyzed by OD_{600} up to 50 h.

2.2.3 Effects of frutalin and compounds on yeast cell growth

To analyze the effect of frutalin and of the known activator of caspase-3 and -7, PAC-1 [56] , and of the known activator of caspase-6, 1541 compound [57], on yeast cell growth, transformed cells were incubated in selective induction medium, as described in section 2.2.2, with different concentrations of recombinant frutalin expressed in *P. pastoris* and purified by SEC (0.01 μM, 0.1 μM, 1 μM and 2.5 μM) or PBS, or with 10, 25, 50 and 100 μM activator or with DMSO only. The time of incubation was the same described in section 2.2.2 according to the protein studied. Yeast cell growth was analyzed after 2 days incubation at 30 °C on Sabouraud Dextrose Agar plates (Liofilchem), by counting the number of colony-forming units (CFU). The percentage of growth was estimated considering as 100 % growth the number of CFU obtained with the control yeast (transformed with the empty vector).

2.2.4 Western Blot Analysis

To analyze protein expression in yeast, samples were lysed in CellyticTM Y Cell Lysis Reagent (Sigma) in the presence of EDTA-free protease inhibitor cocktail (Boehringer Mannheim). Proteins were quantified with Bio-Rad Protein assay (Bio-Rad) and thereafter were electrophoresed on 12% SDS-PAGE (described in section 2.1.5). After this, proteins were transferred to a nitrocellulose membrane (GE Healthcare). Membranes were blocked with Tris- buffered saline (TBS), pH 7.4 (20 mM Tris-base, 150 mM NaCl,) containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat milk at room temperature for 2 h. For detection of procaspase-3, -6 and -7, membranes were probed with anti-procaspase-3 (1:2000, H-277, Santa Cruz Biotechnology), anti-procaspase-6 (1:200, Cell Signaling) and anti-procaspase-7 (1:500, Santa Cruz Biotechnology) monoclonal antibodies, respectively. For loading control, membranes were stripped and incubated with a mouse monoclonal anti-yeast phosphoglycerate kinase (Pgk1p) antibody (1:5000; Molecular Probes). Immunoblots were developed by enhanced chemiluminescence.

2.2.5 Caspase activation analysis

To evaluate the caspase activation by frutalin the fluorescent caspase inhibitor was used (CaspACE, FITC-VAD-FMK *In Situ* Marker; Promega). Yeast cells growth of control yeast and yeast expressing human procaspase-3, -6 and -7 was performed as described in 2.2.3. When the control yeast achieved 0.4 OD_{600}, cells were harvested by centrifugation at 4000 rpm for 5 minutes and washed once with 500 μL PBS. Then, to 1×10^6 cells 500 μL PBS was added and collected by centrifugation at 7000 rpm for 7 minutes. Cells were resuspended in 100 μL PBS-solution containing 12.5 μM FITC-VAD-FMK and incubated for 1 h, at 30 °C in the dark (200 rpm). After this, 400 μL PBS was added to the suspension and the cells were harvested by centrifugation, washed and resuspended in 500 μL PBS. Fluorescence of twenty thousand cells per sample was analyzed using FACSCalibur flow cytometer (BD Biosciences), FL1 channel (Excitation/Emission=488/525 nm) and the CellQuest software (BD Biosciences).

2.3 Assays in human tumor cell lines

2.3.1 Growth conditions of cell culture

In assays with human tumor cell lines the human colon adenocarcinoma HCT116 cell line harboring a wt p53 form (HCT116 $p53^{+/+}$) and its isogenic derivative, in which p53 has been knocked out

(HCT116 $p53^{-/-}$) was used (kindly provided by Dr. B. Vogelstein, The Johns Hopkins Kimmel Cancer Center, Baltimore, MD, USA). Cell lines were routinely cultured in RPMI with ultraglutamine medium (Lonza) supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained in a humidified incubator at 37 °C containing 5% CO_2. When the cells reached the confluence, they were washed with PBS, trypsin (Sigma) was added and they were harvested by centrifugation at 12000 g for 5 minutes. After this, cells were resuspended in RPMI with ultraglutamine medium.

2.3.2 Effect of frutalin on the *in vitro* cell growth of human tumor cells

The anti-proliferative effect of frutalin was evaluated on a human colon carcinoma cell line with (HCT116 $p53^{+/+}$) and without (HCT116 $p53^{-/-}$) wt p53 using the protein-binding dye sulforhodamine B (Sigma) to assess cell growth [58]. Briefly, cells were plated in 96-well plates at a density of 5×10^3 cells/well, in RPMI-1640 medium with ultraglutamine I (Lonza) supplemented with 5% fetal bovine serum (Gibco), and allowed to adhere overnight in a humidified incubator at 37 °C with 5% CO2 in the air. Cells were further incubated for 48 hours with five serial dilutions of recombinant frutalin expressed in *P. pastoris* and in *E. coli*, starting with the concentration of 1.5 μM or 8.4 μM, respectively. PBS was tested with the same volume of the maximum concentration and used as negative control. Following this period, adherent cells were fixed *in situ* with ice cold 10% trichloroacetic acid (TCA; Scharlau) to each well and then were incubated for 1 h at 4 °C. Afterward, plates were washed with distilled water and when plates were well dried, they were stained with SRB solution (0.4% Sulfurhodamine B in 1% acetic acid) and incubated for 30 minutes. Unbound stain was washed with 1% acetic acid and the bound stain was solubilized in 10 mM Tris Base. Finally, optical density was measured at 510 nm using a microplate reader (Synergy HT; Biotek). The GI_{50} values (concentration resulting in 50% inhibition of cell growth) were determined for frutalin from the plotted results.

Chapter 3
Results

3.1 Production and purification of recombinant frutalin

3.1.1 Production and purification of frutalin expressed in *Pichia pastoris*

Recombinant frutalin was expressed in *P. pastoris* as previously described [31]. In order to confirm the correct expression of frutalin, the supernatants of methanol-induced cultures were analyzed by SDS-PAGE and stained with Coomassie. As can be observed in Figure 5 (marked box), frutalin appears as a double band with both bands having a molecular weight higher than 15 kDa, partially glycosylated, as previously reported [31].

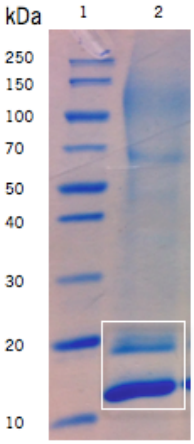

Figure 5. Expression of frutalin in *P. pastoris.* Analysis of supernatants from methanol-induced cultures in a 12% SDS-PAGE stained with Coomassie. Legend: 1, molecular weight standards; 2, frutalin.

After verifying that frutalin was correctly expressed, samples were purified by two different purification techniques: size-exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC). SDS-PAGE analysis of the samples after purification by SEC (Figure 6) was performed in order to confirm that frutalin was obtained at a high level of purity.

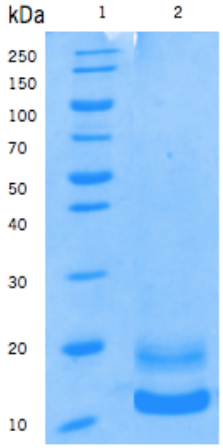

Figure 6. Purification of frutalin expressed in *P. pastoris*. Analyze of frutalin samples obtained from SEC purification by Coomassie blue stained 12% SDS-PAGE. Legend: 1, molecular weight standards; 2, frutalin.

In an attempt to simplify the purification procedure and to decrease its costs, the efficacy of the HIC method was evaluated. In order to assess if it was possible to purify frutalin expressed in *Pichia pastoris* from supernatants of methanol-induced cultures by HIC, proteins were separated on 12% SDS-PAGE and visualized using Coomassie staining (Figure 7A) and silver staining (Figure 7B). For that, two independent assays were performed in order to improve the interaction of the frutalin with the hydrophobic resin. In the assay 1, some target protein was lost in the flow-through and washing step (lanes 3, 4 and 5 of Figure 7A). Nevertheless, a large amount of target protein was recovered in elution step (lane 6 and 7 of Figure 7A). Despite the loss of protein, frutalin showed a high level of purity. In assay 2, the results were more satisfactory than in assay 1 because there was no loss of protein in flow-through and washing step, as it can be seen in Figure 7B. In lanes 12-18 of Figure 7B it is possible to observe a high level of purification of frutalin. However, this only occurred when the elution buffer was PBS (lanes 12-14 of Figure 7B) or 50 mM Tris (lanes 15-18 of Figure 7B). With the remaining of the tested elution buffer (lanes 6-11 of Figure 7B) the elution of frutalin did not occur. For the following studies with human tumor cell lines, the samples shown (Figure 7B) in the lanes 12-15 were mixed and corresponding to the concentrate 1 (white box marked in silver stained SDS-PAGE gel), and the lanes 16-18 were mixed and corresponding to the concentrate 2 (blue box marked in silver stained SDS-PAGE gel) were selected.

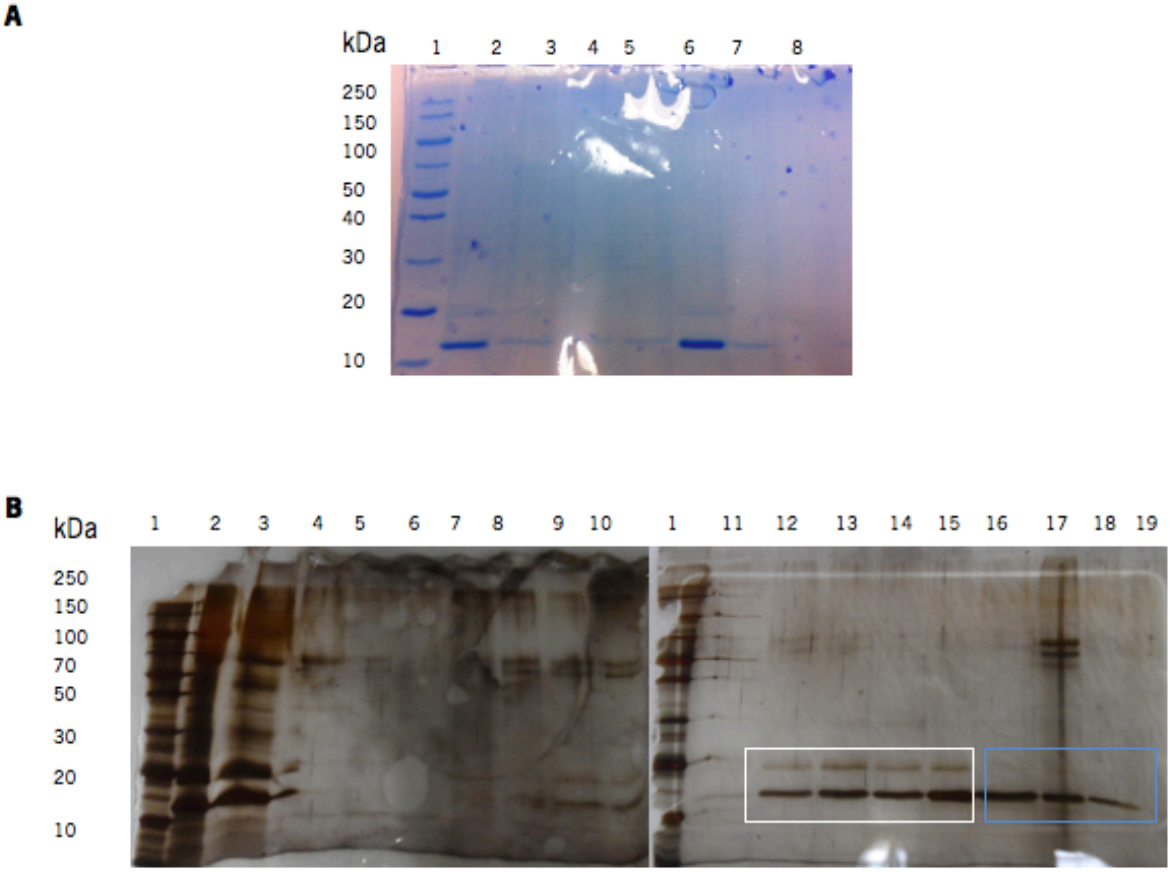

Figure 7. Analysis of recombinant frutalin samples obtained from HIC purification by Coomassie (assay 1) and silver (assay 2) stained 12% SDS-PAGE. (A) Assay 1; Legend: 1, molecular weight standards; 2, frutalin before purification; 3, flow-through sample; 4, washing sample; 5, washing sample using PBS (diluted 1:2); 6-7, eluted samples; 8, washing column using NaOH; (B) Assay 2; Legend: 1, molecular weight standards; 2, FTL before purification; 3, FTL (dilution 1:2 with PBS with 6 M NaCl); 4, flow-through sample; 5, washing sample; 6, eluted sample with PBS with 1 M NaCl; 7-8, eluted samples with PBS with 0.5 M NaCl; 9-11, eluted samples with PBS with 0.25 M NaCl; 12-14, eluted samples with PBS; 15-18, eluted samples with 50 mM Tris (pH 10).

3.1.2 Production and purification of frutalin expressed in *E. coli*

Frutalin was expressed in *E. coli* with two tags: His_6 tag and Fh8 tag. A previous study showed that the molecular weight of Fh8-FTL is approximately 27 kDa [29]. Thus, SDS-PAGE analysis confirmed that Fh8-FTL was expressed correctly and purified with a high level of purity (Figure 8A). In order to confirmed that dialysis of eluted samples obtained from IMAC purification occurred as expected, SDS-PAGE analysis were performed (Figure 8B).

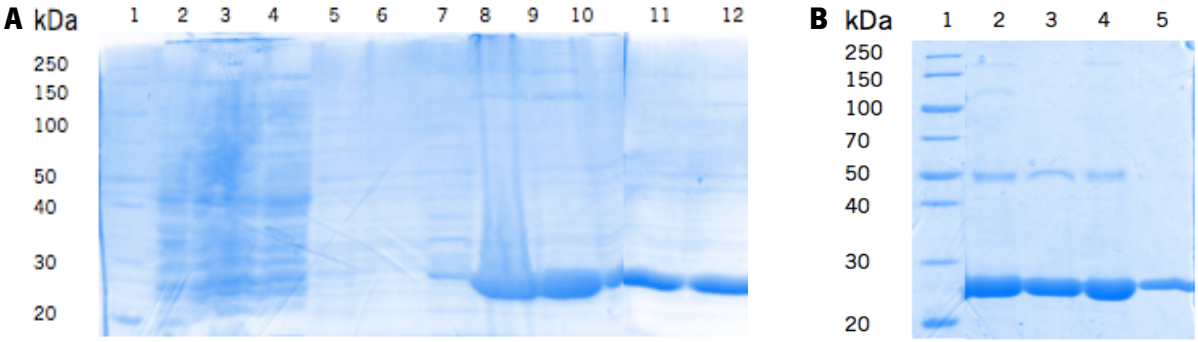

Figure 8. Analysis of Fh8-frutalin samples (A) obtained from nickel affinity purification (IMAC) and (B) after dialysis process by Coomassie stained 12% SDS-PAGE. (A) Legend: 1, molecular weight standards; 2, supernatant sample; 3, flow-through sample; 4-6, washing samples; 7-12, eluted samples. (B) Legend: 1, molecular weight standards; 2-5, Fh8-frutalin samples.

3.2 Yeast- based assay for the elucidation of the molecular mechanism of frutalin

3.2.1 Implementation of a yeast assay to search for procaspase-3, -6 and -7 activators

A previous study showed that recombinant frutalin expressed in *Pichia pastoris* and purified by SEC induced inhibition of cell proliferation in HeLa cells through an apoptotic cell death [33]. Because the p53 family members and the executioner caspases are key apoptotic regulators, we evaluated the effect of frutalin obtained from *P. pastoris* and purified by SEC on these proteins. For that, yeast-based assays were used. For p53 family proteins (p53, p63, p73, DN) these assays, based on growth analysis were already implemented. For the executioner caspases-3, -6 and -7 these assays had to be developed. For that, these caspases were expressed in the yeast *S. cerevisiae* as previously reported [59, 60].

Expression of procaspase-3, -6 and -7 was confirmed by Western blot analysis using specific monoclonal antibodies to each procaspase (Figure 9).

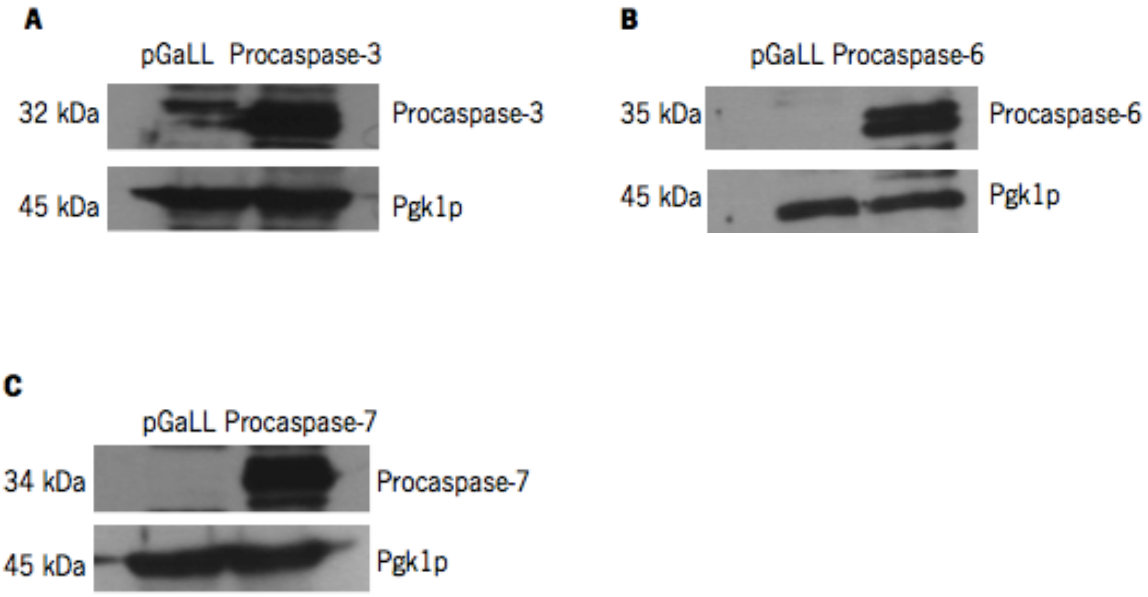

Figure 9. Yeast expression of human procaspase-3 (A), procaspase-6 (B) and procaspase-7 (C) was confirmed by Western blot analysis, using anti-procaspase-3, anti-procaspase-6 and anti-procaspase-7 monoclonal antibodies, respectively; Pgk1p was used as loading control. Immunoblots were developed by enhanced chemiluminescence.

In order to analyze the effect of procaspase-3, -6 and -7 in yeast cell growth, growth curves of yeast cells expressing human procaspase-3, -6 or -7 and control yeast (pGALL) were obtained. A previous study showed that the expression of these human procaspases in yeast did not interfere with the yeast cell growth [61]. The growth curves obtained confirms the previously reported data, since, a similar growth profile was obtained with control yeast and yeast expressing human procaspase-3, -6 or -7 (Figure 10).

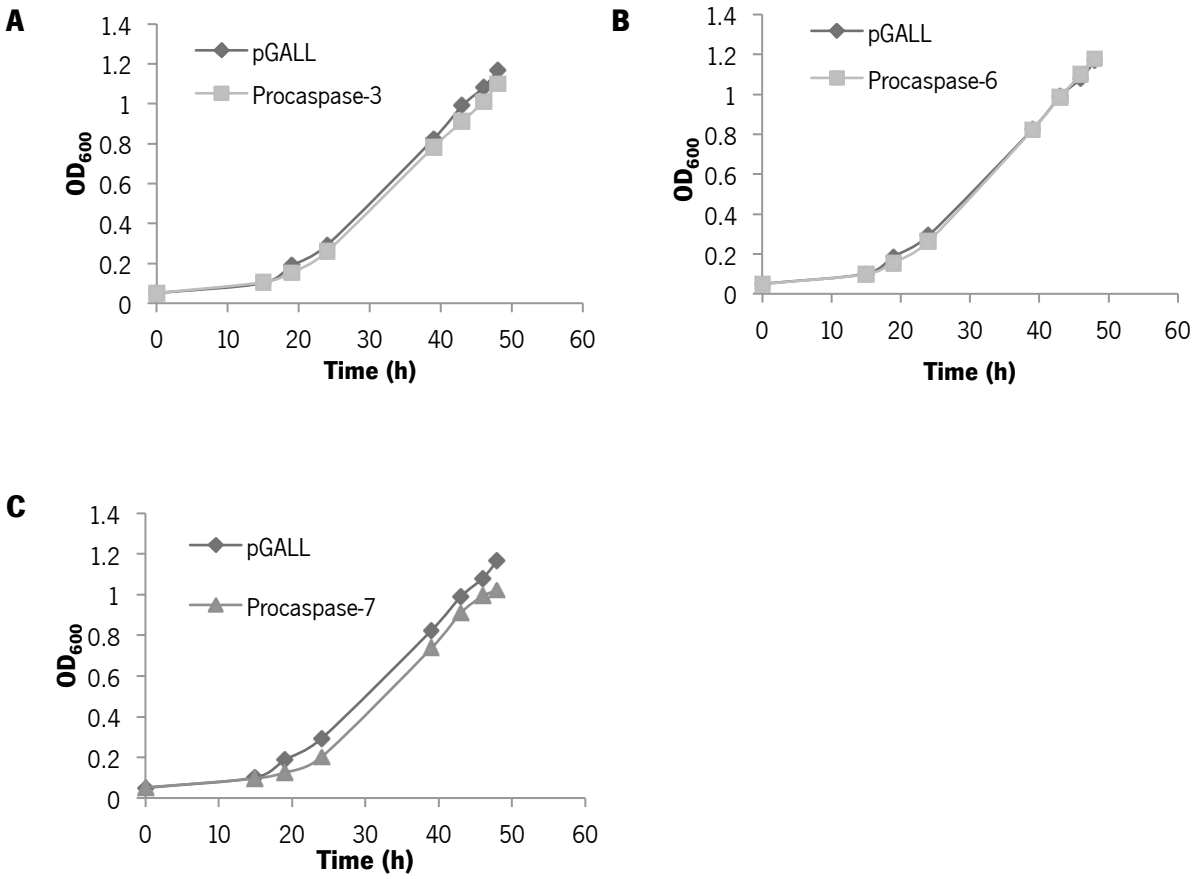

Figure 10. Growth curves for yeast expressing human procaspase-3 (A), -6 (B) and -7 (C) and control yeast (pGALL) assessed by optical density (at 600 nm). Data represent mean ± standard error (S.E.M) of two independent experiments; values obtained not statistically different from yeast expressing control empty vector ($P > 0.05$).

To validate these assays for the search of activators, the commercial activators of executioner caspases, PAC-1 and compound 1541, were tested. As previously described, PAC-1 is a known activator of procaspase-3 and -7 and the compound 1541, a known activator of caspase-6 [56, 57]. As expected, a decrease on yeast cell growth to all the procaspases studied, can be observed in response to the standard activators. However, for procaspase-6 this growth inhibition was not statistically significant (Figure 11).

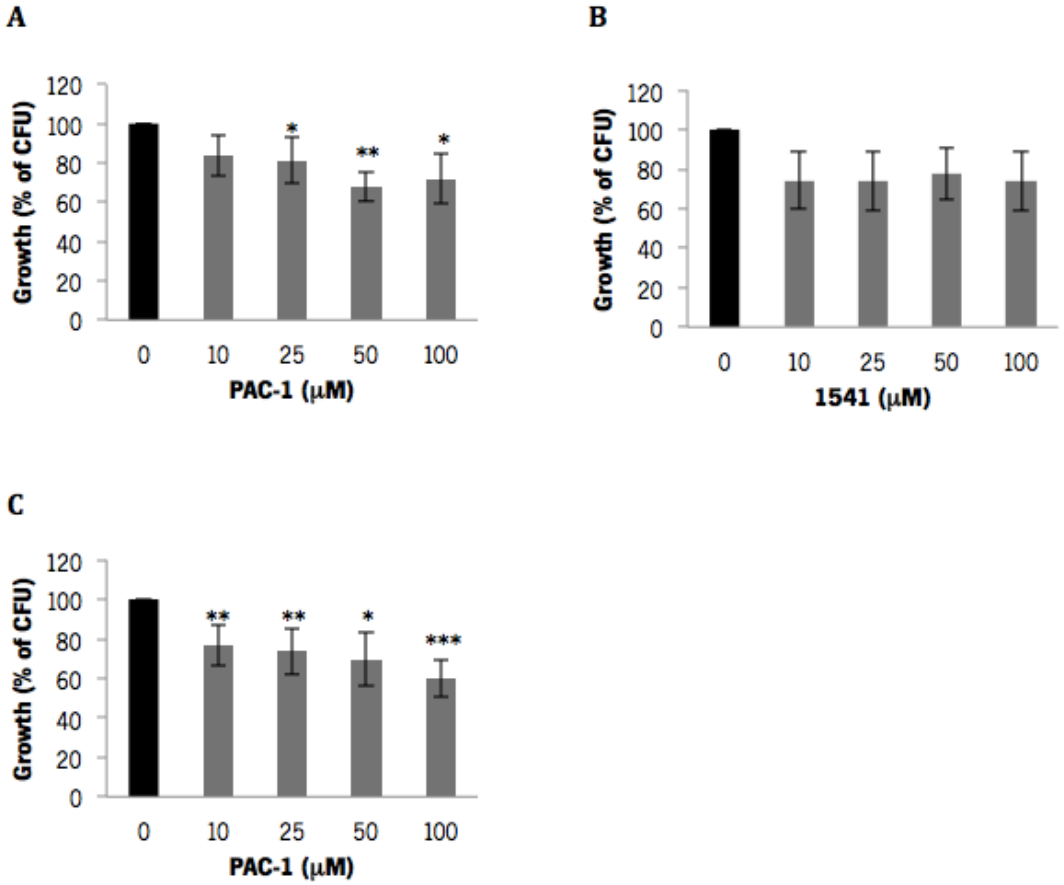

Figure 11. (A) Effect of PAC-1 on the growth of yeast expressing procaspase-3 (B) Effect of the compound 1541 on the growth of yeast expressing procaspase-6 (C) Effect of PAC-1 on the growth of yeast expressing procaspase-7. Yeast cells expressing executioner procaspases were incubated in the presence of 10, 25, 50 and 100 μM activator compound or DMSO only, for 48 h incubation. For each culture, the percentage of drug-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with control yeast (pGALL). Data represent mean ± S.E.M (n=3 for procaspase-3; n=2 for procaspase-6 and n=4 for procaspase-7); values significantly different from DMSO only (**P*<0.05; ***P*<0.01; ****P*<0.001).

3.2.2 Effect of frutalin on key apoptotic regulators using yeast assays

Yeast assays were performed to analyze the effect of frutalin expressed in *Pichia pastoris* and purified by SEC in the activity of several apoptotic regulators. These assays are based on the fact that these apoptotic regulators induce growth inhibition when expressed and activated in yeast. Yeast cells transformed with the empty vector (control) or expressing p53, p63, p73 or DN were incubated with 0.1 μM or 1 μM frutalin for 30 h. We observed that frutalin did not interfere with the growth of yeast (Figure 12).

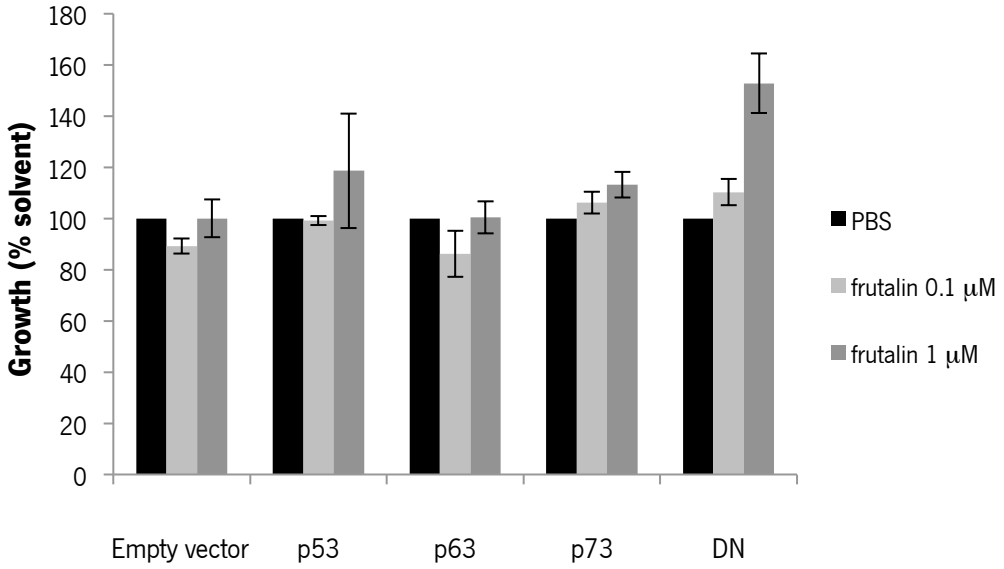

Figure 12. Effects of different concentrations of frutalin on the growth of control yeast and yeast expressing p53, p63, p73 or DN. For each culture, the percentage of frutalin-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with PBS only. Data represent mean ± S.E.M of three independent experiments; values obtained not statistically different (P> 0.05).

The treatment of yeast cells expressing procaspase-3 with 0.1 and 1 μM of frutalin, markedly decreased the growth of cells (Figure 13). Although it was observed a growth inhibition induced by frutalin with 0.01 μM, 0.1 μM and 1 μM in yeast cells expressing procaspase-7, this was not statistically significant. Additionally, no effect of frutalin was observed on the growth of yeast expressing procaspase-6 (Figure 13). The results obtained for procaspase-3 suggest that this procaspase may be involved in the apoptotic pathway triggered by frutalin.

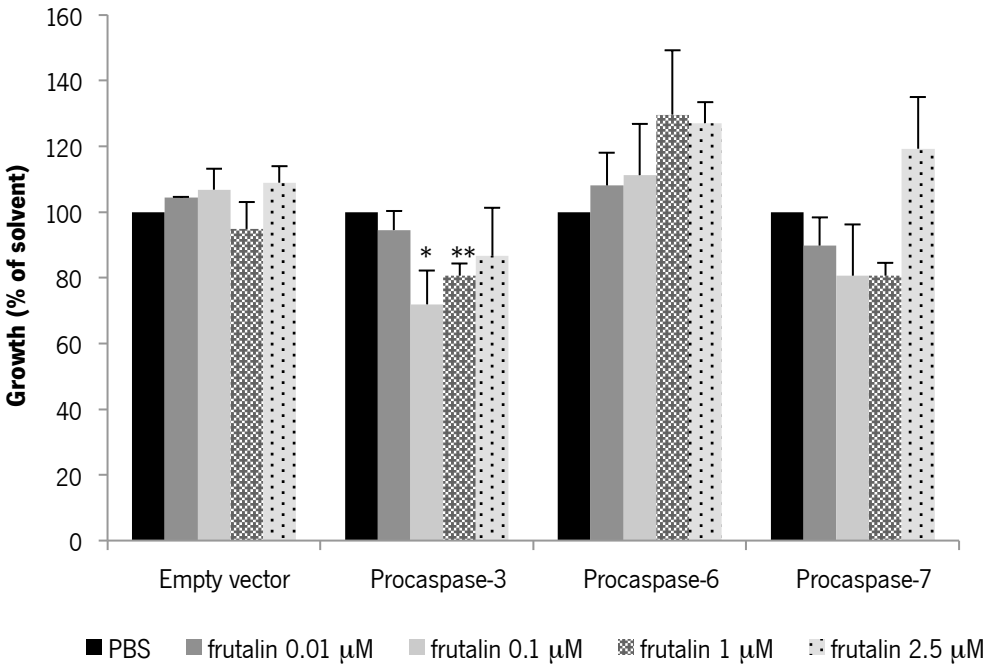

Figure 13. Effects of different concentrations of frutalin on the growth of control yeast (empty vector) and yeast expressing procaspase-3, procaspase -6 and procaspase-7, for 42 h treatment. For each culture, the percentage of frutalin-induced growth inhibition was estimated considering 100% growth the number of CFU obtained with PBS only. Data represent mean ± S.E.M of three independent experiments; values obtained with yeast expressing procaspase-3 treated with frutalin 0.1 μM and frutalin 0.1 μM significantly different from PBS (*P< 0.05; ** P<0.01).

Additionally, the effect of frutalin on procaspase-3, -6 and -7 activation was assessed using the assay CaspACE FITC–VAD–FMK in yeast. This assay consists in the use of a fluorogenic substrate that binds to active caspases. The expression of procaspase-3, -6 and -7 incubated only with the solvent PBS already exhibit some caspace activity, which may reflect some basal auto activation (Figure 14). In the presence of 0.01 μM, an increase in the activity of 14.94%, 19.05% and 27.67% was observed for yeast cells expressing procaspase-3, -6 and -7, respectively. A slight increase of caspases activity was also observed in the empty vector, which may reflect the activity of yeast endogenous proteases (Figure 14). In this assay, the values obtained showed a higher activation of procaspase-3 and -7 (50.50% and 48.66%, respectively) than procaspase-6 (33.55%).

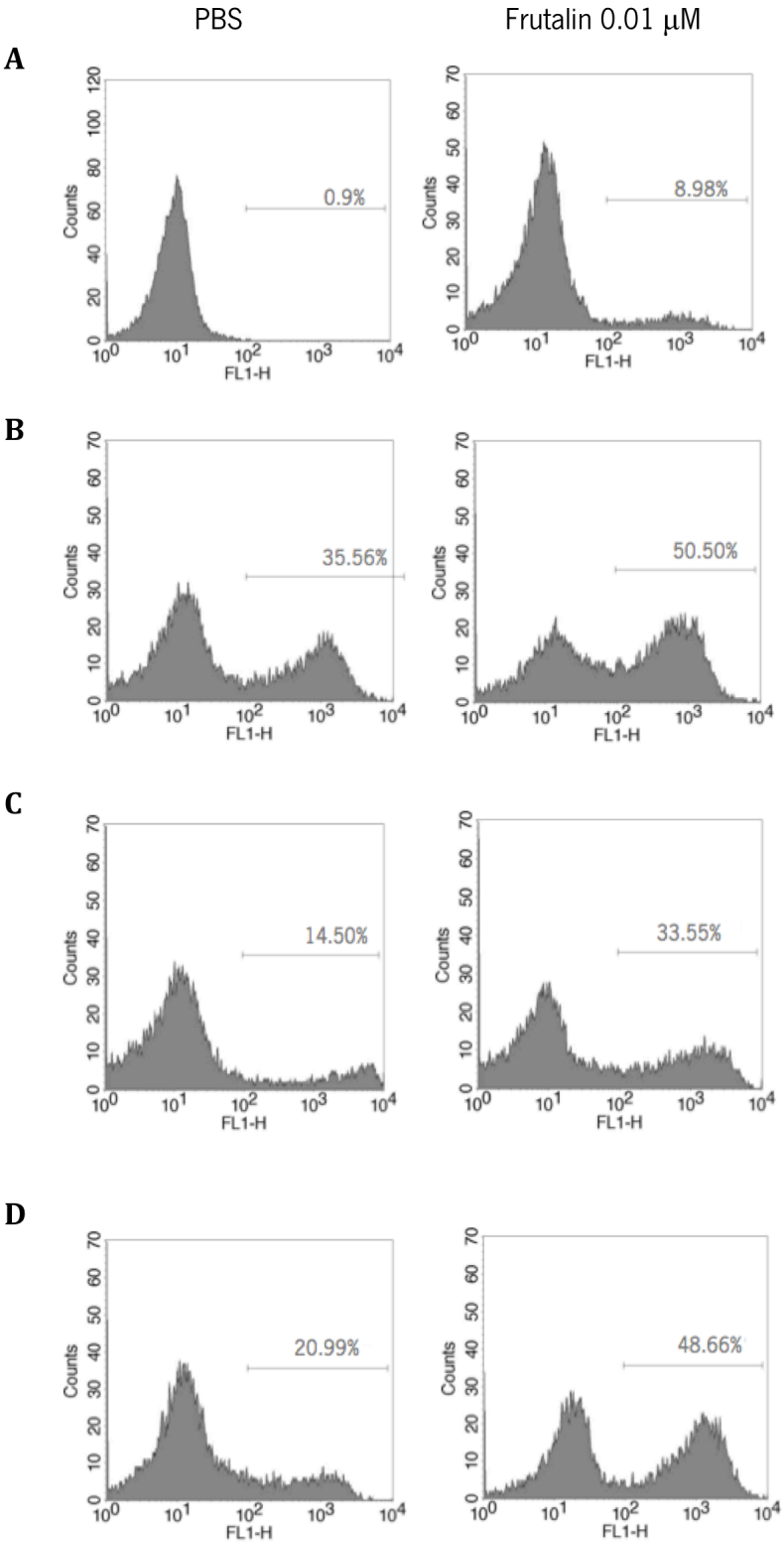

Figure 14. Procaspase activity in yeast after treatment with frutalin. Flow cytometric analysis using the FL1 detection of FITC fluorescence of cells treated with PBS (control) and cells treated with 0.01 µM of frutalin. Data represent one experiment with duplicates. Legend: (A) Empty-vector (B) Procaspase-3 (C) Procaspase-6 (D) Procaspase-7.

3.3 Studies in human tumor cell lines

In order to confirm the results obtained in yeast, that suggest that the p53 family is not involved in the induction of apoptosis by frutalin, the effect of frutalin expressed in *Pichia pastoris* and purified by SEC in human tumor cells of colon adenocarcinoma HCT116 cell line with (HCT116 $p53^{+/+}$) and without (HCT116 $p53^{-/-}$) wt p53, was evaluated. The effect of frutalin in the inhibition of cell proliferation was similar in both HCT116 cell lines analyzed (Figure 15; Table 5). These results confirmed those obtained in yeast, indicating that frutalin does not induce cell death by activation of the p53 pathway.

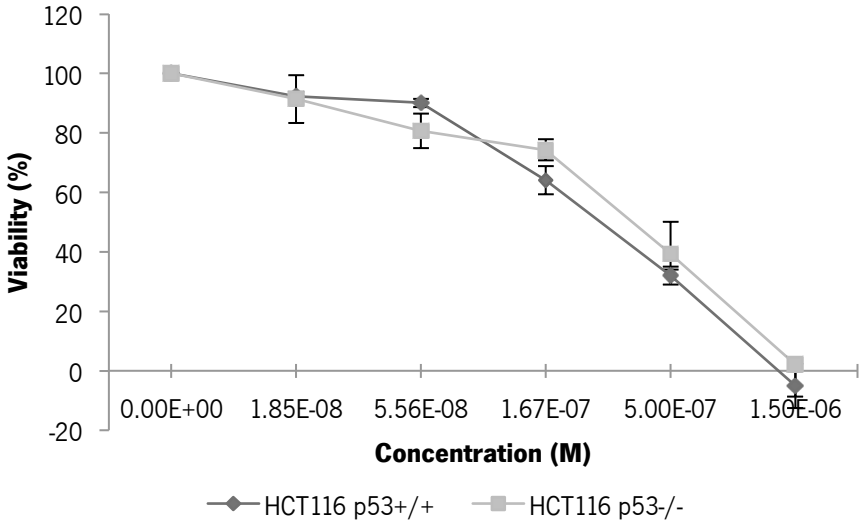

Figure 15. Effect of different concentrations of frutalin on proliferation of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells at 48 h. Data represent mean ± S.E.M of two independent experiments; values obtained are not statistically different (P >0.05).

We also tested the frutalin expressed in *P. pastoris* and purified by HIC (that resulted in two different concentrates as can be observed in Figure 7B). These concentrates were analyzed in human tumor cells with wt p53 (HCT116 $p53^{+/+}$) to evaluate if this purification affects the biological activity of this protein. The GI_{50} value determined for concentrate 1 was higher than that determined for frutalin expressed in *P. pastoris* purified by SEC (frutalin used in the assays of this work). Concerning to the concentrate 2 none of the concentration tested in tumor cells caused inhibition of cellular proliferation.

The effect of frutalin expressed by *E. Coli* (Fh8-FTL) was also evaluated in human tumor cells with wt p53 (HCT116 $p53^{+/+}$) and none of the concentrations tested (0.5 μM, 1.1 μM, 2.1 μM, 4.2 μM and 8.4 μM) showed inhibition of cell proliferation.

Table 5. GI_{50} of frutalin obtain in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells

Frutalin/ Tumor cell lines	**Frutalin (Purified by SEC)** GI_{50} (μM) ± S.E.M	**Frutalin (purified by HIC)** GI_{50} (μM)	
		Concentrate 1	Concentrate 2
HCT116 $p53^{+/+}$	0.27 ± 0.037	0.69	High (> 0.55)
HCT116 $p53^{-/-}$	0.45 ± 0.083	-	-

Data represent mean ± S.E.M of two independent experiments.

Chapter 4
Discussion

Frutalin, a α-D-galactose-binding lectin, was previously shown to have inhibitory activity on the proliferation of tumor HeLa cells. In the present work recombinant frutalin was produced and purified from *Pichia pastoris* and *Escherichia coli*.

The expression of frutalin in *P. pastoris* occurred as expected [31]. A double band was observed in SDS-PAGE and according to *Oliveira et al.* [31], the molecular weight obtained on SDS-PAGE confirming that the expression of frutalin occurred without excision of linker tetrapeptide, as a single chain protein. Moreover, the upper band represents the β-linker-α chain of the glycosylated isoforms wherein the lower band corresponds to the β-linker-α chain of the non-glycosylated isoforms [31]. In order to purify frutalin expressed in *P. pastoris* two different purification techniques were performed: size-exclusion chromatography and hydrophobic interaction chromatography. According to the previously described [31], the purity level of frutalin using SEC was high. The HIC purification by performing two assays with different buffers composition was optimized. Concerning to the first assay, some protein was lost in flow-through and washing samples. These may be due to the weak interaction between the hydrophobic surface of the sample and the hydrophobic ligands of a HIC medium. This suggests that frutalin weakly exposed the hydrophobic amino acids residues probably due to lower concentrations of salts in buffers [62, 63]. Therefore, in the second assay, the concentration of salts in the sample and in the start buffer was increased. Thus, it was expected a stronger exposure of the hydrophobic amino acids residues and, consequently, a better interaction between the surface of the sample and the hydrophobic resin [62–64]. As expected, it was verified a better interaction since, frutalin was not lost in flow-through and in washing samples. Moreover, in this second assay, frutalin showed a high level of purity.

Concerning the expression of Fh8-frutalin in *E. coli*, this protein migrated as a single band on SDS-PAGE. The Fh8-frutalin, show a molecular weight of approximately 27 kDa. Contrary to the frutalin expressed in *P. pastoris*, the frutalin expressed in *E. coli* is non-glycosylated. This protein showed a high level of purity through nickel affinity purification (IMAC).

Interestingly, the concentrates (1 and 2) of pure frutalin obtained from *P. pastoris* and purified by HIC (second assay) showed different results. The concentrate 1 had a potent anti-proliferative effect against tumor cells with wt p53 (HCT116 $p53^{+/+}$). In opposition, the concentrate 2 had no anti-proliferative effect against tumor cells with wt p53 (HCT116 $p53^{+/+}$). In SDS-PAGE, the lanes correspondents to this concentrate do not present the upper band that represents the β-linker-α chain of the glycosylated isoforms. However, the intensity of the lower band that corresponds to the β-linker-α chain of the non-glycosylated isoforms is similar to the concentrate 1. This suggests that the concentrate 2 is non-

glycosylated. The β-linker-α chain of the glycosylated isoforms is present for concentrate 1 and for frutalin purified by SEC. Thus, the absence of anti-proliferative effect for concentrate 2 may be due to the lack of glycosylation in the purified frutalin. Glycosylation is very important for the physical and functional properties of proteins. Thus, the changes in glycosylation of frutalin may have led to changes in the structure and biological activity of this protein [65, 66]. In the purification of frutalin by HIC, the concentration of salts in the medium was increased. These salts enhances the changes in the protein conformation in order to expose the hydrophobic residues and thus, to interact with hydrophobic ligands of the HIC medium [64]. These changes in the structure can lead to the loss of biological activity [64–66]. Thus, the decrease of the activity of concentrate 1 (when compared to the frutalin in *P. pastoris* and purified by SEC) may be also due to changes in the frutalin conformation, although in a lower degree than that observed with concentrate 2. The absence of anti-proliferative effect of the concentrate 2 it also can be due to changes in the frutalin conformation. Concerning to frutalin expressed from *E. coli*, this had no anti-proliferative effect in inhibition of cellular proliferation on HCT116 $p53^{+/+}$ tumor cells. As concentrate 2, frutalin expressed in *E. coli*, is also non-glycosylated, exhibiting a similar effect to this concentrate. Thus, the results obtained with concentrate 2 and frutalin from *E. coli* also suggest that the absence of glycosylation contributed to the loss of frutalin biological activity, nevertheless, further experiments should be conducted to confirm this hypothesis.

In a previous study, it was verified that frutalin expressed in *P. pastoris* and purified by SEC induced inhibition of growth cell by apoptosis [33]. *Oliveira et al.* [33] reported that this frutalin had a potent anti-proliferative effect on HeLa cell line ($GI_{50} \approx 100$ μg/mL ≈ 5.85 μM). In the present work, frutalin was tested in human colon adenocarcinoma HCT116 cell lines. Similarly, a potent anti-proliferative activity was observed for frutalin expressed in *P. pastoris* and purified by SEC against HCT116 tumor cells.

As potential drugs in future cancer therapeutics, the understanding of the molecular mechanism involved in lectin-induced apoptosis in tumor cells is widely required.

According to the results obtained in yeast assays, frutalin seems to interfere with the caspase pathway, particularly with the procaspase-3. However, the results obtained in the flow cytometric analysis also showed the activation of procaspase-6 and -7. This may be due to the small number of assays performed in growth assays and in flow cytometric analysis. Thus, further assays should be performed in order to confirm if only procaspase-3 is activated. Despite of these, the results suggest that the anti-proliferative activity of frutalin is mediated through a caspase-dependent pathway. Furthermore, the yeast assays indicated that frutalin was not able to activate p53 family proteins. Additionally, studies in human

tumor cell with (HCT116 $p53^{+/+}$) and without (HCT116 $p53^{-/-}$) wt p53, confirmed that p53 was not involved in frutalin-induced apoptosis. These results were consistent with previous data showing that some lectins such as *Astragalus membranaceus* lectin (AML), Concanavalin A (Con A), *Polygonatum odoratum* lectin (POL) and *Polygonatum cyrtonema* lectin (PCL) induce apoptosis in tumor cells in a caspase-dependent pathway. Interestingly, FTL, AML, Con A, POL, and PCL have different carbohydrate-binding specificity [47, 67–69]. Additionally, lectins such as jacalin and the *Cliona varians* lectin (CvL), which as frutalin bind specifically to galactose, induce apoptosis in a caspase-independent pathway [70, 71]. The carbohydrate-binding specificity of lectins is very important for their biological activities. However, and according to previous data obtained for frutalin and other lectins, it seems that the biological effects of lectins are not only dependent on the carbohydrate-binding property [72, 73]. Differences in anti-proliferative activity of lectins in tumor cell lines have been reported, confirming that biological effects of lectins not depend only of their sugar specificity. A previous report, demonstrate that Galβ1-3Gal-NAcα- binding lectin (jacalin) was noncytotoxic inhibitor of proliferation of HT29 colon cancer cells however, was cytotoxic to A431 epidermoid carcinoma cells [72]. AML induces different responses in growth of K562 and HeLa cell line and was more inhibitor of proliferation in the K562 cell line [74].

Chapter 5
Conclusions and future perspectives

This work aimed to elucidate the molecular mechanism involved in induction of apoptosis by frutalin expressed in *Pichia pastoris* and purified by size-exclusion chromatography. Therefore, using yeast-based assays the major proteins involved in apoptosis such as caspases executioner and p53 family proteins were studied. Moreover, the effect of this frutalin in the proliferation of human tumor cells with (HCT116 $p53^{+/+}$) and without a wt p53 (HCT116 $p53^{-/-}$) was evaluated. The results obtained suggest that frutalin induced apoptosis in a caspase-dependent pathway and that the p53 pathway was not involved in frutalin-induced apoptosis.

Additionally, we analyzed the effect of frutalin expressed in *Escherichia coli* on the proliferation of HCT116 $p53^{+/+}$ tumor cells, and showed that this frutalin has no effect in the cell proliferation. Thus, even though the production and purification process in *E. coli* be less time consuming and more straightforward, this expression system should not be used for the production and purification of frutalin. Nevertheless, the absence of biological activity could be due to the tag presence. Therefore, tag removal should be considered and the obtained cleaved frutalin should be evaluated for its effect in the proliferation of human tumor cells so that a definitive conclusion can be reached on the use of this bacteria. Frutalin expressed in *P. pastoris* was also purified by hydrophobic interaction chromatography that result in two different samples, suggesting that one is partially glycosylated and the other is non-glycosylated. The sample partially glycosylated inhibited cell proliferation of HCT116 $p53^{+/+}$ tumor cells however, less potent than frutalin purified by SEC whereas the sample non-glycosylated did not inhibit cell proliferation.

As future work, it would be interesting to explore the caspase-dependent pathway since this is the probable mechanism triggered by frutalin to induce apoptosis. Thus, it would be necessary to confirm the activation of procaspase-3, -6 and -7 by frutalin by western blot analysis in human tumor cell lines. Human cell lines silenced for these caspases would also validate these proteases as targets of frutalin. Additionally, *in vitro* assays, using recombinant caspases would confirm if these proteases are direct targets of frutalin, as suggested by the yeast assays.

Chapter 6
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Chapter 7
Appendices

7.1 Amino-acid sequence of Frutalin expressed in *Pichia pastoris*

NQQSGKSQTVIVGPWGAKVSTSSNGKAFDDGAFTGIREINLSYNKETAIGDFQVVYDLNGSPYVGQNHKSFITGFTPV
KISLDFPSEYIMEVSGYTGNVSGYVVVRSLTFKTNKKTYGPYGVTSGTPFNLPIENGLIVGFKGSIGYWLDYFSMYLSL

7.2 Amino-acid sequence of His-Frutalin expressed in *Escherichia coli*

MKHHHHHHPMSDYDIPTTENLYFQGAMGSAEQSGKSQTVIVGPWGAKVSTSSNGKAFDDGAFTGIREINLSYNKET
AIGDFQVIYDLNGRPFVGQSHTSFIKGFTPVKISLDFPSEYIVEVSGHTGKVSGYVVVRSLTFKTNKKTYGPYGVTSGTPF
NLPIENGLVVGFKGSIGYWMDYFSMYLSL

7.3 Amino-acid sequence of Fh8-Frutalin expressed in *Escherichia coli*

MKHHHHHHPMSPSVQEVEKLLHVLDRNGDGKVSAEELKAFADDSKCPLDSNKIKAFIKEHDKNKDGKLDLKELVSI
LSSENLYFQGSMGSAEQSGKSQTVIVGPWGAKVSTSSNGKAFDDGAFTGIREINLSYNKETAIGDFQVIYDLNGRPFVG
QSHTSFIKGFTPVKISLDFPSEYIVEVSGHTGKVSGYVVVRSLTFKTNKKTYGPYGVTSGTPFNLPIENGLVVGFKGSIGY
WMDYFSMYLSL

Legend:

- Beta chain
- Linker tetrapeptide
- Alpha chain
- His_6 tag
- Fh8 tag